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Research Article

Evaluation of Agrobacterium-mediated Transformation of *Chlamydomonas reinhardtii* Using a Synthetic Amorpha-4, 11-diene Synthase Gene

Afsaneh Mohkami[1*], Hassan Marashi[1], Farajolah Shahriary Ahmadi[1], Masoud Tohidfar[2] and Motahareh Mohsenpour[2]

1. Biotechnology and Plant Breeding Department, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
2. Tissue Culture and Gene Transformation Department, Agriculture Biotechnology Research Institute of Iran (ABRII), Karaj, Iran

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Abstract

Amorpha-4,11-diene synthase (ADS) is a key enzyme in biochemical pathway of the antimalarial agent artemisinin. An Agrobacterium-mediated transformation was carried out to express a synthetic *ADS* gene in green microalga *Chlamydomonas reinhardtii* strain 125C with bacterial strains GV3101 and LBA4404. The foreign gene was optimized based on codon usage bias of the microalga. Integration of the *ADS* in nuclear genome of *C. reinhardtii* was confirmed by polymerase chain reaction assay. The transgenic colonies cultured on selective medium turned yellow after three days and gradually died. Transformation procedure, growth habit of the transgenic microalgae together with probable causes of transformants loss is discussed. The present study is the first investigation for production of ADS enzyme in a microalgal system.

Keywords: Amorpha-4,11-diene synthase, *Chlamydomonas reinhardtii*, Agrobacterium and Transformation

Introduction

In many regions of the world especially in developing countries malaria is a prevalent infectious disease which is the tenth cause of death and is predicted to remain at that level until at least 2030 (Castello et al., 2010). It is estimated that more than 380 million cases of malaria occur annually accounting for more than 1 million deaths (Briolant et al., 2010).

The most potent treatment for malaria disease is an herbal compound called artemisinin and its derivatives. Artemisinin is a secondary metabolite produced in the herbaceous plant *Artemisia annua*. Artemisinin has a short half-life so significant quantities in prescribed doses is required and thus there is an increasing demand for this important therapeutic agent (Crockett et al., 2007). Moreover, although artemisinin is still the preferred treatment for high risk patients, its use is hampered by its high cost, because is produced a very low amount of this compound in *A. annua*. Enhancing artemisinin concentration in plant tissues is, therefore, a practical way to make artemisinin available as a cheap antimalaria drug (Wallaart et al., 2000). An ideal approach to achieve this goal would be genetic engineering of enzymes involved in biosynthesis of artemisinin. Artemisinin is synthesized in plant tissues via a complex pathway including different enzymes, the most critical of which is amorpha-4,11-diene synthase (ADS) which catalyzes conversion of farnesyl diphosphate (FPP) in to amorpha-4,11-diene (Arsenault et al., 2008).

Considering low content of artemisinin in *A. annua* tissues and high cost of synthetic production of the product on one hand, and increasing demand for artemisinin on the other hand; there have been extensive investigations for expression of artemisinin complex precursors such as Amorpha-4,11-diene in microbial platforms such as *E. coli* and *Saccharomyces cerevisiae* and even in the higher plant tobacco (*Nicotiana tabacum*) aiming at achieving a cost-effective way for production of artemisinin (Khosla et al., 2003).

Although production of artemisinin precursors in bacterial and yeast hosts has been successful, large scale production of artemisinin in such systems which requires vitamin and carbon source is not

Corresponding authors E-mail:
* afi_mohkami@yahoo.com, af_mo360@stu.um.ac.ir

cost effective (Walker et al., 2005). This limitation can be circumvented by adopting an appropriate host such as fresh water microalgae *Chlamydomonas reinhardtii*.

Application of microalgae for production of pharmaceuticals offers a number of advantages not found in other production systems. Microalgae are cost effective platforms for therapeutics production and are free of human or animal pathogens (Dove, 2002; Tran et al., 2009). Moreover, they possess elaborated cellular machinery required to fold complex human proteins that bacteria and yeast may not be able to process properly (Rosenberg et al., 2004). Many species of green algae are generally regarded as safe (GRAS) microorganisms, meaning that if the protein can be expressed in a bioavailable form, purification steps could potentially be eliminated altogether. This will result in further reduction of production costs (Rasala et al., 2010).

Microalgae can be grown in contained bioreactors, reducing the risk of contamination of the production system by airborne contaminants, and also protecting the environment from any potential flow of transgenes into the surrounding ecosystem. Time course from initial transformation of microalgae to large-scale protein production is only a matter of weeks, compared to months or years in higher plants (Franklin and Mayfield, 2004). Indeed, algal-based platforms combine the advantages of microbial and plant systems for production of therapeutic agent (Rasala et al., 2010).

Genetic transformation of microalgae can be carried out in many ways including microparticle bombardment, glass beads, electroporation and Agrobacterium-mediated transformation (Soria-Guerra et al., 2013). Since the pioneer work of Kumar et al (2004), Agrobacterium-mediated transformation has been widely used for genetic manipulation of microalgae. This method is a simple and yet efficient approach that can be carried out in a relatively short time with low cost (Specht et al., 2010).

The goal of the present study was to investigate production of amorpha-4,11-diene as a major precursor of artemisinin in *C. reinhardtii* using Agrobacterium-mediated genetic transformation.

Materials and Methods

Construct design

Nucleotide sequence of *ADS* gene was obtained from NCBI website (http://www.ncbi.nlm.nih.gov/) with accession number of AF138959.1 and the corresponding protein sequence was obtained with accession number of AAF61439.1. The coding sequence was optimized using Vector NTI advance 11 software. This sequence encodes for the 63.933 kD ADS protein. To facilitate cloning procedure in pBI121 plasmid, *Bam*HI and *Sac*I restriction sites were included in 5´ and 3´ ends of the gene, respectively. The *ADS* gene was synthesized in pGEM vector (Bioneer, south Korea).

Construction of expression vector

The synthetic *ADS* gene was removed from pGEM vector by digestion with *Bam*HI and *Sac*I and inserted into the plant expression vector pBI121, downstream of the CaMV 35S promoter and upstream of the nopaline synthase (NOS) terminator, yielding pBI121-ADS vector. *Bam*HI/*Sac*I double digestion removes *GUS* gene from pBI121 plasmid.

The resulted vector was used to transform *E. coli* strain DH5-α and kanamycin-resistant colonies were selected after overnight incubation at 37°C. For confirmation of the recombinant plasmid pBI121-ADS, at the first the plasmid was extracted from bacterial cells using alkaline lysis method, then digestion of recombinant plasmid with *Eco*RI and PCR analysis of the recombinant plasmid using gene specific-forward primer and NOS terminator-specific reverse primer was performed. The sequence of forward and reverse primers: 5' TAAATGGGCTAATGAAGGTC 3' and 5' GAAGAAAGCGAAAGGAGC 3', respectively. PCR was carried out as follow: 94 $^{\circ C}$ 45s, 52 $^{\circ c}$ 45s, 72 $^{\circ c}$ 1.5 min, 30 cycles.

pBI121-ADS vector was introduced into *Agrobacterium tumefaciens* strain GV3101 and LBA4404 by thaw-melting method. Transformed cells were selected on screening medium (LB medium containing 50 mg/l kanamycin and 50 mg/l rifampicin) and colony PCR was carried out similar to that already explained.

Chlamydomonas culture

Chlamydomonas strain 125C was obtained from Chlamydomonas Resource Center (University of Minnesota). Algal cells were grown in Tris-Acetate-Phosphate (TAP) medium under continuous cool fluorescent light at 25°C.

Genetic transformation of *C. reinhardtii*

C. reinhardtii cells were transformed using the method proposed by Kumar et al (2004) with some modifications. In summary, 10^7 *Chlamydomonas* cells were transferred on to the solid TAP medium with 100 µM and without acetosyringon (AS) and incubated in light for 2 days to form algal lawn. A fresh *Agrobacterium* culture (A600 = 0.5) grown in

liquid LB medium containing appropriate antibiotics (50 mg/l rifampicin and 50 mg/l kanamycin) was centrifuged in 4500rpm for 10 min, the supernatant was discarded and the bacterial pellet was resuspended in 200 µl TAP broth with or without AS. The bacterial suspension was spread to the thin layer of *Chlamydomonas* lawn growing on agar plate. The co-cultivation plates were incubated for 2 days at 25°C. The cells were then harvested and washed twice with liquid TAP medium containing 500 mg/l cefotaxime to eliminate bacterial residues and centrifugation at 1000 rpm for 2 min, resuspended in liquid TAP. The washed *Chlamydomonas* cells were cultured in solid selection medium containing 50 mg/l Kanamycin and 500 mg/l cefotaxime.

PCR screening of transformants

Half of each transformant colony was screened for presence of the gene of interest using aforementioned primers. *Sac*I digestion was carried out to confirm PCR products. Cells were resuspended in Tris-EDTA solution and heated to 95°C for 10 minutes. The cell lysate was used as a template for PCR reactions. The other halves of the colonies with positive PCR results were grown in liquid selection medium containing 50 mg/l Kanamycin for further analysis**.**

Results

Design and construction of expression cassette

After codon optimization of *ADS* gene and inclusion of *Bam*HI and *Sac*I restriction sites in 3´ and 5´ ends, the optimized 1653 nucleotide sequence was synthesized and cloned in pGEM vector. After digestion of pGEM-ADS and pBI121 vectors with abovementioned enzymes, the *ADS* gene was inserted in pBI121 vector (figure1).

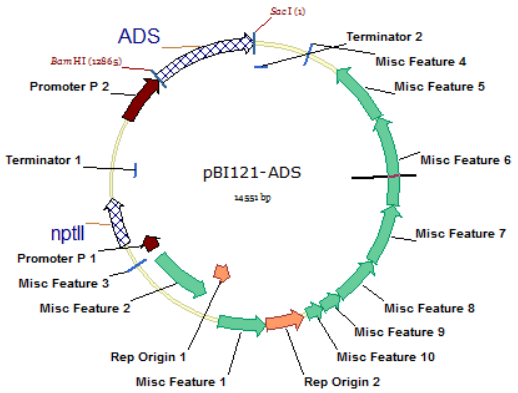

Figure 1. Schematic components of pBI121-ADS expression vector

After transfer of pBI121-ADS in to *E.coli* cells, the extracted plasmid of the putative transformed cells were evaluated using digestion with *Eco*RI restriction enzyme (figure 2) and PCR with specific primers (figure 3). *Eco*RI endonuclease has two restriction sites on the recombinant plasmid; one on pBI121 plasmid and the other on *ADS* gene.

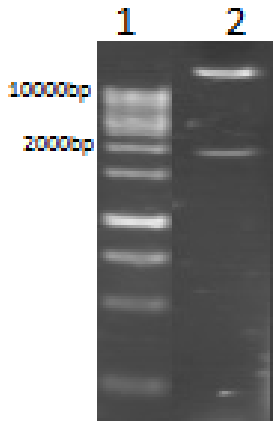

Figure 2. Digestion product of *Eco*RI. lane1: 1kb ladder (Fermentas); lane 2: digestion pBI121-ADS with *Eco*RI (12600bp and 1900 bp segments).

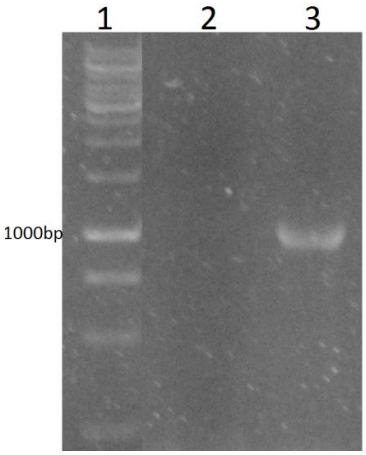

Figure 3. lane1: 1kb ladder (Fermentas); lane 2: negative control (*E.coli*); lane 3: Transformed cell (The 1000bp band resulting from PCR with specific primers)

Genetic transformation of *C. reinhardtii*

Genetic transformation of *C. reinhardtii* was carried out using two strains of *A. tumefaciens* namely LBA4404 and GV3101. The transformed colonies appeared on selective medium after 7-10 days. In the lack of acetosyringone treatment, 2-3 colonies were obtained for both strains of Agrobacterium. However, by application of acetosyringone (100 µM), the number of transformants increased significantly, that is 270 and 350 colonies were achieved for LBA4404- and GV3101-mediated transformation, respectively.

PCR assay

Polymerase chain reaction (PCR) was performed to evaluate presence of foreign gene in putative transgenic microalgal cells. For each strain of Agrobacterium, twenty colonies were used for colony PCR among which, seven colonies showed the expected band (figure 4). In evaluation of PCR product using digestion with *Sac*I restriction enzyme, desired segments was observed. (figure 5)

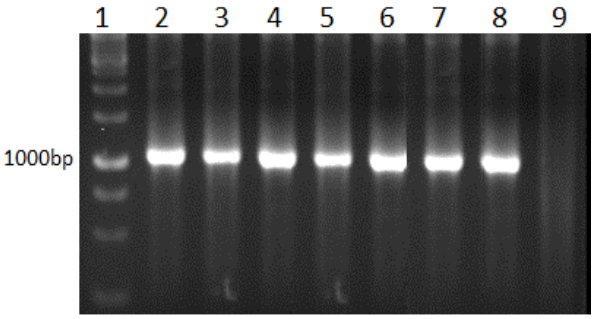

Figure 4. Gel electrophoresis of colony PCR products. Lane 1: 1kb ladder (Fermentas); lanes 2-5: colonies transformed using GV3101; 6-8: colonies transformed using LBA4404 and lane 9: wild type

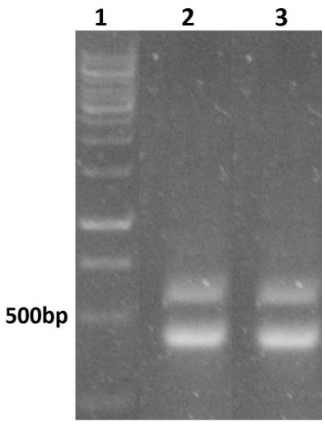

Figure 5. Digestion of PCR product with *Sac*I. lane1: 1kb ladder (Fermentas); lane 2-3: digestion of PCR product with *Sac*I (600bp and 400bp segments).

Growth kinetic of transformant colonies

As stated in Material and methods section, half of each transgenic colony was cultured in selective broth medium. The colonies showed normal growth in the first three days creating a bright green color; however the cells got yellow thereafter and were completely lost on the seventh day. The experiment was repeated for more colonies but the results were the same. Some colonies even showed no growth in selective medium. The lack of growth in selective liquid media prevented from subsequent analyses such as expression assay.

Discussion

In this study, we investigated application of green microalgae *C. reinhardtii* as a platform for expression of ADS as a key enzyme in biochemical pathway of artemisinin. Although heterologous expression of ADS has been reported in other biological systems (Arsenault et al., 2008), to the best of our knowledge the present study is the first investigation for production ADS enzyme in a microalgal system. The synthetic *ADS* gene was optimized based on codon usage of *C. reinhardtii*. It has been reported that low expression of foreign genes in algal system is often due to the incompatibility of the codon usage in their coding regions which consequently decreases the efficiency of the translation (Mayfield et al., 1990).

Although we were not able – due to loss of transgenic lines- to analyze the recombinant protein in translation level, some results achieved in this investigation worth noting. Acetosyringone had significant influence on the percentage of transformants, increasing from 2-3 colonies in 0µM to 350 colonies in 100µM of acetosyringone. This is in accordance with the results reported by Kumar et al (2004) who found out that 100µM of acetosyringone significantly enhanced transformation percentage.

Application of GV3101 strain led to better results compared to LBA4404 strain. Similar to our results, in a recent investigation it was reported that GV3101 was effective in increasing transformation rate (Habibi-pirkoohi et al, 2014). However, in another study investigating efficacy of different strains of *A. tumefaciens* in genetic transformation of microalgae, the best results were achieved by strain EHA 105 (Pratheesh et al., 2012). It seems that adoption of a certain strain as the best candidate for transformation of microalgae is not conclusive.

Another feature of the present study was instability of transformed cells of *C. reinhardtii*. The green colonies of transformed cells gradually turned in to yellow about two weeks after transformation procedure. Instability of microalgal transformed colonies has been reported by other authors (Leon et al., 2007; Habibi et al., 2014). Generally, in chlorophytes such as *C. reinhardtii*, transformation frequency of heterologous constructs is very low and gene expression is instable (Neupert et al., 2009). The unique nuclear characteristics of these microalgae can influence their ability to express genes under the control of heterologous promoters (von Gromoff et al., 2008).

It is generally accepted that in chlorophytes and diatoms, stable expression of heterologous genes

can only be optimally achieved when adequate homologous promoters or promoters from very close species are included (Shao and Bock, 2008).
A great number of regulatory elements and transcriptional or post transcriptional events can influence on the expression level of the transgenes and on their stability (Kathiresan et al., 2009). Expression of an exogenous gene can be very low or null, even though all the elements required for optimal transcription and translation -promoters, introns and other regulatory regions- have been included in the chimeric gene construction. Difficulties for foreign gene expression in microalgae can be due to the lack of adequate regulatory sequences, to positional effects, biased codon usage, incorrect polyadenylation, inappropriate nuclear transport, instability of the mRNA, or gene silencing (Rasala et al., 2010).
In the present study, the transformed colonies were cultured on selective media; whereas in the pioneer work conducted by Kumar et al (2004), the transformants were cultured in non-selective medium. It has been reported that when transgenic algal clones are not maintained under selection conditions, expression of the exogenous genes might be suppressed (Leon et al., 2007). Furthermore, to verify reliability of the results reported by Kumar et al (2004), we cultured some PCR-positive clone in non-selective media. The colonies underwent normal growth, but after full growth no band was observed for these colonies in PCR assay, suggesting that the transformants lost the transgene during growth in non-selective medium. The results obtained in the present investigation can be justified by transient expression theory. Transient expression of firefly luciferase (Luc) in protoplasts of *Chlorella ellipsoidea* and of β-glucuronidase (*Gus*) gene in *C. saccharophila* has been already reported (Leon et al., 2007).
It can be postulated that expression of ADS exerts a metabolic load on *C. reinhardtii* cells and this suppresses microalgal growth. However, since the growth of transformants on selective media was normal for a few days, the hypothesis of metabolic load can't be so reliable. Therefore, we believe that instable gene expression is the probable cause of the results observed in the present study.

As a conclusion, agrobacterium-mediated procedure is a simple and cost-effective method for nuclear transformation of microalgae and an acceptable number of colonies is achieved in this way. However, stable expression of ADS- and other foreign genes- in *C. reinhardtii* using agrobacterium-mediated transformation requires more elaborated constructs and algae-specific regulatory elements specially homologous promoters (Spetch et al., 2010).
Although gene expression was not analyzed in this investigation, based on the same results obtained in duplicate experiments and similar number of colonies achieved with two different Agrobacterium strains, it can be concluded that further studies along with application of homologous elements are required to achieve appropriate and stable results in nuclear transformation of *C. reinhardtii* moreover, it appears that combating gene silencing will be a major hurdle before recombinant proteins can be expressed at economically viable levels from nuclear transgenes in *Chlamydomonas*. (Specht et al., 2010).

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